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Netrins and DCC in the guidance of migrating neural crest-derived cells in the developing bowel and pancreas

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Abstract

Vagal neural crest-derived precursors of the enteric nervous system colonize the bowel by descending within the enteric mesenchyme. Perpendicular secondary migration, toward the mucosa and into the pancreas, result, respectively, in the formation of submucosal and pancreatic ganglia. We tested the hypothesis that netrins guide these secondary migrations. Studies using RT-PCR, in situ hybridization, and immunocytochemistry indicated that netrins (netrins-1 and -3 mice and netrin-2 in chicks) and netrin receptors [deleted in colorectal cancer (DCC), neogenin, and the adenosine A2b receptor] are expressed by the fetal mucosal epithelium and pancreas. Crest-derived cells expressed DCC, which was developmentally regulated. Crest-derived cells migrated out of explants of gut toward cocultured cells expressing netrin-1 or toward cocultured explants of pancreas. Crest-derived cells also migrated inwardly toward the mucosa of cultured rings of bowel. These migrations were specifically blocked by antibodies to DCC and by inhibition of protein kinase A, which interferes with DCC signaling. Submucosal and pancreatic ganglia were absent at E12.5, E15, and P0 in transgenic mice lacking DCC. Netrins also promoted the survival/development of enteric crest-derived cells. The formation of submucosal and pancreatic ganglia thus involves the attraction of DCC-expressing crest-derived cells by netrins.

Keywords: Chemoattraction; Intestine; Enteric nervous system; Pancreatic ganglia; Submucosal plexus

Introduction

The enteric nervous system (ENS) is unique in its ability to function independently of CNS control and in its resemblance to the CNS (Furness, 2000; Gershon, 1995, 1998, 1999; Gershon et al., 1994). The ENS is derived from precursors that migrate to the gut from the neural crest (Le Douarin and Teillet, 1973, 1974; Yntema and Hammond, 1954, 1955). Vagal crest-derived precursors migrate proximodistally through the outer mesenchyme of the presumptive small intestine (Burns and Le Douarin, 1998, 2001; Coventry et al., 1994; Kapur et al., 1992; Natarajan et al., 1999; Young et al., 1998). Most of these cells form myenteric ganglia; however, a subset migrates perpendicularly toward the mucosa and forms the submucosal plexus (Gershon et al., 1993; McKeown et al., 2001; Payette et al., 1984), while another subset migrates in the opposite direction out of the gut to form pancreatic ganglia (Kirchgessner et al., 1992). The guidance factors responsible for determining the routes of crest-derived cell migration in the bowel have not previously been identified.

Netrins attract or repel sets of axons, usually to or from the midline, but also elsewhere, in worms, flies, and vertebrates (Culotti and Merz, 1998; Serafini et al., 1994; Wang et al., 1999). Netrins are members of a family of laminin-related proteins, which include the UNC-6 gene product of nematodes, netrins-A and -B of *Drosophila*, netrins-1 and -2 of chicks, netrin-3 of mice, and the NTN2L gene product of humans. The chemoattractant effects of netrins are mediated by the deleted in colorectal cancer (DCC) family of plasmalemmal receptors, which includes the UNC-40 protein of nematodes, the Frazzled protein of *Drosophila*, and DCC and neogenin in vertebrates. The adenosine A2b receptor also binds netrins, and evidence for (Corset et al., 2000) and against (Stein et al., 2001) the idea that it acts with DCC as

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a coreceptor has been reported. Netrins function, not only in axonal guidance, but also in neuronal migration and survival in the ventricular zone of the brainstem (Llambi et al., 2001), inferior olive (Bloch-Gallego et al., 1999), and pontine nuclei (Serafini et al., 1996). Whether effects of netrins are attractive or repulsive depends on coreceptors, such as the UNC-5 gene product, with which DCC interacts (Barrett and Guthrie, 2001; Hong et al., 1999), and the level of cAMP within navigating growth cones (Hopker et al., 1999; Ming et al., 1997).

Migrating crest-derived cells and the growth cones of spinal axons respond to some of the same signals. Netrin-3, moreover, has been found in the developing murine PNS (Seaman and Cooper, 2001). We therefore tested the hypothesis that netrins are expressed in the developing gut and pancreas and function in the guidance of DCC-expressing enteric crest-derived cells. Our observations suggest that the netrin-dependent attraction of DCC-expressing crest-derived cells plays a critical role in the formation of the submucosal plexus of the bowel and ganglia in the pancreas.

Materials and methods

Animals

Fertile eggs from White Leghorn chickens (*Gallus gallus*) and quails (*Coturnix coturnix japonica*) were obtained from Truslow Farms (Chestertown, MD) and Karasoulas Farms (Lake Elsinore, CA), respectively, and incubated at 37°C. CD-1 mice and timed pregnant rats were obtained from Sprague-Dawley (Charles River, Waltham, MA) and killed by exposure to CO_2. This procedure has been approved by the Animal Care and Use Committee of Columbia University. Rodent pregnancies were timed from the day a vaginal plug was discovered, which was considered E0. Fetuses from transgenic mice lacking DCC (Fazeli et al., 1997) were from the laboratory of Dr. M. Tessier-Lavigne (Department of Biological Sciences, Howard Hughes Medical Institute, Stanford University).

Immunoselection

The monoclonal antibody, HNK-1, was either prepared from the supernatants of cultured hybridoma cells (American Type Culture Collection, Manassas, VA) or purchased from Biomeda (Foster City, CA). HNK-1 is a marker for crest-derived cells in the chick (Tucker et al., 1984, 1988; Vincent et al., 1983; Vincent and Thiery, 1984) and rat (Erickson et al., 1989) gut. These antibodies were used to isolate crest-derived and non-crest-derived cells from the fetal gut by positive and negative immunoselection (Chalazonitis et al., 1997a; Pomeranz et al., 1993). Guts were pooled from 15–20 chick embryos (E6), transferred to a Ca^{2+}/Mg^{2+}-free solution (137 mM NaCl, 5.3 mM KCl, 1.1 mM Na_2HPO_4, 1.1 mM KH_2PO_4, 0.6 mM D-Glucose, pH 7.2–7.4), and minced. The minced tissue was incubated with collagenase A (5 mg/ml; Roche Molecular Biochemicals, Indianapolis, IN) at 37°C for 30 min, followed by trituration and filtration to produce a suspension of single cells. The resulting cell suspension was subsequently incubated for 60 min at 4°C with a 1:50 dilution of HNK-1 antibodies. The incubation was terminated by a brief rinse with serum-free medium. The rinsed cells were then exposed at 4°C for 15 min to goat anti-mouse secondary antibodies coupled to magnetic microbeads (Miltenyi Biotec Inc., Auburn, CA). The antibody-decorated cells were then passed through a column in which they were selected with a magnetic field. The crest-derived cells are retained on the column by the magnetic field while the residual non-crest-derived cells pass through. The crest-derived cells were finally released from the magnetic field and washed out of the columns.

Tissue culture

Explants

Segments of preumbilical bowel distal to the gizzard were removed from E5 chick or quail embryos and cut transversely to obtain thin, but complete, rings of tissue. The resulting enteric rings were plated on 12-mm coverslips coated with NH_4OH-polymerized rat tail collagen. Explants of pancreas were prepared by cutting the dorsal pancreatic bud away from the foregut, taking care not to include bowel. When explants of gut and pancreas were to be cocultured, the enteric explants were first incubated for 30 min at 37°C with a marker, 5-chloromethylfluoroscein diacetate (CellTracker Green; 5 μM in αMEM; Molecular Probes, Eugene, OR) to allow migrating crest-derived cells from the bowel to be traced. The labeled rings of bowel were then plated on 12-mm collagen coverslips, and the explants of pancreas were plated next to them at a distance of 300–600 μm. The individual explants and the explants of CellTracker-labeled gut + pancreas were cultured in an explant medium containing 10% horse serum and 2% chick embryonic extract (CCE), in DMEM for 40 h.

Expression of netrin-1 in stably transfected cells

Control HEK293 cells (InVitrogen, Carlsbad, CA) and HEK293 cells stably transfected with a construct encoding netrin-1 tagged at the C terminus with a myc epitope (supplied by Dr. M. Tessier-Lavigne) (Keino-Masu et al., 1996) were plated on plastic in petri dishes and maintained in a medium consisting of DMEM supplemented with 10% heat-inactivated fetal bovine serum, 0.5% glutamine and pen/strep, 200 μg/ml Hygomycin B (Roche Molecular Biochemicals), and 250 μg/ml Geneticin (G418 sulfate; Gibco BRL, Life Technologies). The transfected cells HEK293, but not the HEK293 controls, could be immunostained with antibodies to Myc (monoclonal 9E10; Santa Cruz), verifying that the transfected cells expressed the netrin-1 construct. Control and transfected HEK293 cells were harvested by rapid flushing and resuspended in DMEM

Table 1
Primary antibodies utilized in the current study

Antigen	Species	Dilution	Source
PGP9.5 (ubiquitin hydrolase)	Rabbit	1:500	Biogenesis Ltd. Poole, United Kingdom
HNK-1	Mouse	1:1000	Biomeda Corp., Foster City, CA
QCPN	Mouse	1:5	Monoclonal antibody developed by M. Bruce and Jean Carlson was obtained from the Developmental Studies Hybridoma Bank, University of Iowa, under NIH contract HD-7-3263 (Liem, 1995)
Mouse glucagon	Rabbit	1:200	Chemicon International, Temecula, CA
Chick pan-netrin	Rabbit	1:100	Prepared in the laboratory of M. Tessier-Lavigne, University of California, San Francisco, CA (Deiner et al., 1997)
Human DCC	Mouse	1:100	Oncogene, Cambridge, MA (Deiner et al., 1997)
Mouse netrin-1	Goat	1:100	Santa Cruz Biotechnology, Santa Cruz, CA
Mouse DCC	Goat	1:200	Santa Cruz Biotechnology, Santa Cruz, CA
Mouse A2b adenosine receptor	Goat	1:200	Santa Cruz Biotechnology, Santa Cruz, CA
Digoxigenin	Sheep	1:500	Roche Molecular Biochemicals, Indianapolis, IN

containing 10% heat-inactivated fetal bovine serum. Drops of the resuspended cells (~20 μl; 5,000,000 cells/ml) were placed onto the lids of 35-mm culture dishes and inverted over dishes that contained 2 ml DMEM. The resulting hanging drops were incubated for 2 days at 37°C. Aggregates of cells were then harvested with a 200-μl pipette for use in cocultures with explants of gut or dissociated enteric cells. The harvested aggregates of netrin-1-secreting and control 293-EBNA cells were finally embedded in rat-tail collagen (Coulter et al., 1988) for coculture either with explants of gut or with dissociated enteric cells. For coculture with explants of bowel, aggregates of 293-EBNA cells were positioned ~300–600 μm from the gut and a microdrop of diluted rat tail collagen (5 μl, diluted 1:10 in αMEM; Upstate Biotechnology, Lake Placid, NY) was added to cover both. The explants and the 293-EBNA cells were thus both embedded in a three-dimensional collagen gel.

Culture of immunoselected cells from the embryonic chick gut

Positively and negatively immunoselected cells were separately plated (6.0×10^4 cells per well) on 12-mm round glass coverslips coated with poly-D-lysine (20 μg/ml; Sigma) and rat-tail collagen. Cultures of mixed cells dissociated from the embryonic bowel without prior immunoselection were also separately plated and treated similarly. The crest-derived (positively selected) and non-crest-derived (negatively selected) obtained by immunoselection and the mixed cells were then maintained for 40 h in a medium consisting of DMEM supplemented with 4.0% horse serum. To assess the effects of netrin-1 on the cultures of dissociated enteric cells, they were cocultured with collagen-embedded 293-EBNA cells, which were or were not (control) expressing netrin-1. The position of the 293-EBNA cells was thus fixed, while the dissociated enteric cells spread randomly over the dish. Alternatively, enteric cells were cultured for 16 h in the same reduced-serum medium; however, the medium was supplemented with purified netrin-1 (Serafini et al., 1994). Cultures were fixed for analysis by immunocytochemistry to terminate the experiments.

Immunocytochemistry and histochemistry

Freshly removed chick embryos, fetal mice, dissected segments of bowel, or cultures were fixed for 3 h at room temperature or overnight at 4°C in a solution containing 4% formaldehyde (from paraformaldehyde) in 0.1 M sodium phosphate buffer (pH 7.4). All preparations, except the cultures (which were processed as whole mounts), were rinsed with the same buffer, infiltrated with 30% sucrose, and rinsed. They were then embedded in OCT medium (Lipshaw), frozen in liquid N_2, sectioned (at 10 μm) with a cryostat-microtome, and thaw-mounted on chromium alum-gelatin-coated glass slides. Tissues were permeabilized and blocked by incubation in PBS containing 0.4% Triton X-100 and 4.0% horse serum. Primary antibodies (Table 1) were applied overnight in a humidified chamber at 4°C. Sites of antibody binding were detected by incubation for 2 h at room temperature with appropriate secondary antibodies labeled with fluorescein isothiocyanate (FITC), cyanine-3 (Cy3), biotin, or alkaline phosphatase (Table 2). Biotinylated secondary antibodies were detected by the ABC method (Elite kit; Vector Laboratories, Burlingame, CA). Peroxidase activity was visualized with H_2O_2 and 3, 3′-diaminobenzidinesubstrate (DAB). Alkaline phosphatase activity was demonstrated with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in a buffer containing 50 mM $MgCl_2$, 1% Tween 20, 100 mM NaCl, and Tris–HCl at pH 9.5. For double labeling, primary antibodies raised in different species were employed and the species-specific secondary antibodies were coupled to contrasting fluorophores. To enhance the antigenicity of netrins in the chick gut, sections, mounted on slides, were submerged in 0.01 M Na citrate (pH 6.0) and

Table 2
Secondary antibodies utilized in the current study

Antibody	Coupled to	Ig	Dilution	Visualized	Source
Goat anti-mouse	Cy3	IgG	1:1000	Fluorescence	Jackson ImmunoResearch, West Grove, PA
Goat anti-rabbit	Cy3	IgG	1:1000	Fluorescence	Jackson ImmunoResearch, West Grove, PA
Goat anti-rabbit	FITC	IgG	1:400	Fluorescence	Jackson ImmunoResearch, West Grove, PA
Goat anti-rabbit	biotin	IgG	1:200	ABC/DAB	Vector Laboratories, Burlingame, CA
Donkey anti-rabbit	Cy3	IgG	1:500	Fluorescence	Jackson ImmunoResearch, West Grove, PA
Donkey anti-goat	Cy3	IgG	1:1000	Fluorescence	Jackson ImmunoResearch, West Grove, PA
Donkey anti-goat	FITC	IgG	1:400	Fluorescence	Jackson ImmunoResearch, West Grove, PA
Rabbit anti-sheep	biotin	IgG	1:200	ABC/DAB	Vector Laboratories, Burlingame, CA
Horse anti-mouse	Alkaline phosphatase	IgG	1:200	NBT/BCIP	Vector Laboratories, Burlingame, CA

boiled for 10 min prior to incubation with primary antibodies (Deiner et al., 1997). Acetylcholinesterase (AChE) activity was demonstrated histochemically as previously described (Blaugrund et al., 1996; Karnovsky and Roots, 1964).

RNA isolation and RT-PCR

Total RNA was isolated from chick and mouse gut (RNA STAT-60; Tel-Test Inc., Friendswood, TX), and DNA contamination was removed with RNase-free DNase I (Promega, Madison, WI) for 30 min at 37°C. Then, 2 μg of total RNA was used to synthesize cDNA with random primers and MuLV reverse transcriptase (GeneAmp RNA PCR kit; Applied Biosystems, Foster City, CA) in 25 μl of reaction mixture at 42°C for 30 min. The PCR mixture (25 μl) consisted of 5 μl cDNA, 3.75 pmol each of 3′ and 5′ primers, 0.25 mM dNTP, 2.5 μl PCR buffer, and 1.0 U of Taq polymerase. cDNA was amplified in a thermal cycler (Inotech Biosystems, Lansing, MI) set with the following parameters for all primer pairs: denaturation at 95°C (5 min initial cycle, 1 min each subsequent cycle for 30 cycles), annealing at a temperature noted below for 40 s, and extension at 60°C for 1.0 min each cycle for 30 cycles. The sense and anti-sense primers, Mg^{2+} concentrations, and annealing temperatures used are listed in Table 3. The polymerase chain reaction (PCR) was carried out by using the following primers: chick netrin-1, chick netrin-2, mouse netrin-1, mouse netrin-3, chick neogenin, mouse DCC, and β actin. After the final PCR cycle, the reaction was extended for an additional 10 min at 72°C, and the reaction products were then cooled to room temperature. PCR products were resolved by electrophoresis through a 1% agarose gel and visualized with ethidium bromide (0.3 μg/ml). The PCR products were sequenced to confirm their identity. To do so, PCR products were subcloned into a pCR II cloning vector by using a commercial kit (TA Cloning Kit; Invitrogen, Carlsbad, CA). Plasmid DNA was isolated (Wizard Minipreps, Promega) and sequenced by dye termination (ABI Automated Sequencer, Perkin Elmer) in the core facility of Columbia University. The PCR products corresponding to chick netrin-1 and chick netrin-2 were identified by digestion at appropriate sites with the *Ava*II and *Pst*I restriction enzymes. The final cDNA sequences were compared with those in the GenBank (Blast search at National Center for

Table 3
PCR primers utilized in the current study

Proteins	Antisense primer	Sense primer	(bp)	Mg^{2+}/°C
Used with RT-PCR				
β-actin	5′-GTGGGCCGCTCTAGGCACCAA-3′	5′-CTCTTTGATGTCACGCACACGATTTC-3′	508	2 mM/60
Mouse netrin-1	5′-TGACTGTAGGCACAACACGG-3′	5′-CTCCATGTTGAATCTGCAGC-3′	153	2 mM/60
Mouse netrin-3	5′-TGGCTGGTTGACTTACAGCG-3′	5′-ACAAGAGCGATGGTCCCTC-3′	152	2 mM/58
Mouse DCC	5′-CCAAGGTCGTCATGGAGATG-3′	5′-TGGGCACTTTCTAGTGTTGG-3′	620	2.5 mM/60
Mouse neogenin	5′-TACACTCCAGTGCCAGATCC-3′	5′-GCCGGGTACAAAAGACAGCA-3′	856	2.5 mM/60
Chick netrin-1	5′-CAAGGGGAAGCTGAAGATCA-3′	5′-TCTTCATGGGCTTCACCTTG-3′	223	2.5 mM/60
Chick netrin-2	5′-ATCAACCCGACCTCTCTTGT-3′	5′-TGCTGATCTGGATTTTGGGG-3′	288	2.5 mM/60
Chick neogenin	5′-TCTAGCACCAGCTACAACGG-3′	5′-GGATTTCCCCATTGCCAGAC-3′	421	2.5 mM/60
Used with quantitative PCR				
Mouse DCC	5′-CCAACACTAGAAAGTGCCCA-3′	5′-AGTTGCTTCATTAGCCCTTC-3′	556	2.5 mM/60
Chick netrin-1	5′-AGGGAGAAGAAAGGGAAGTG-3′	5′-GGCAGAAGGTTTTGCTCAAG-3′	412	2.5 mM/60
Chick neogenin	5′-CCCAAGATGTTGTTGTGCGG-3′	5′-TCGGAACTTCAGGAACACGG-3′	362	2.5 mM/60
Chick A2b	5′-ACTTCCGCTACACCTTCCAC-3′	5′-TGAACTGCACTCAGTCTCCC-3′	326	2.5 mM/60
Used to clone cDNA encoding DCC for in situ hybridization				
Mouse DCC	5′-GAAAGTGCCACCACTCGTTC-3′	5′-TGGGCACTTTCTAGTGTTGG-3′	1351	3 mM/60

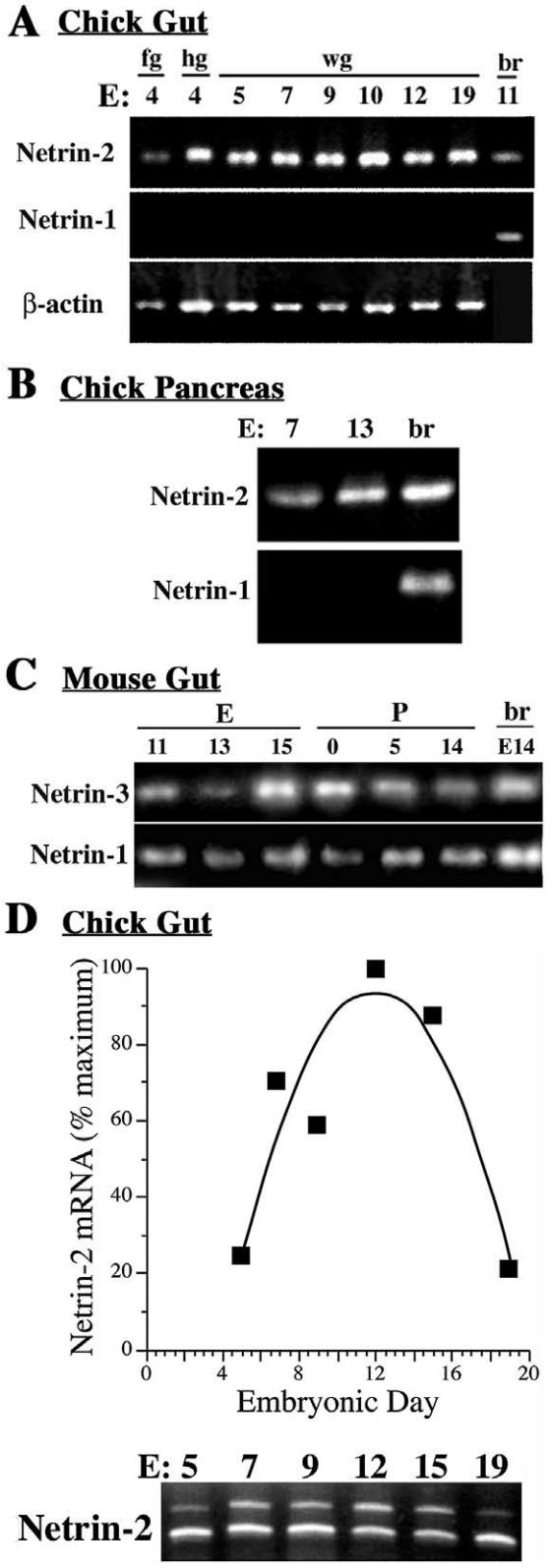


Fig. 1. mRNA encoding netrins can be found by RT-PCR in the developing gut of chicks and mice. (A) Chick gut. mRNA encoding netrin-2 but not netrin-1 is found. E, the age of development at which the gut was analyzed; fg, foregut; hg, hindgut; wg, whole gut; br, brain (analyzed as a positive control). (B) Chick pancreas. As in the bowel, mRNA encoding netrin-2 but not netrin-1 can be detected. (C) Mouse gut. E, ages of fetal mice; P, ages of postnatal mice. (D) Chick gut. Competitive PCR. The expression of netrin-2 in the developing bowel is developmentally regulated and peaks at E12.

Biotechnology Information, Bethesda, MD) to confirm their identity.

Competitive PCR was used to compare quantitatively the expression of netrin-1, DCC, neogenin, and the adenosine A2b receptor at a variety of ages (Uberla et al., 1991). Competitor DNA fragments for quantitative PCR were obtained by using mouse-specific primers to amplify DNA from chicks and chick-specific primers to amplify DNA from mouse under low-stringency annealing conditions (45°C; 30 cycles). These artificially created fragments match the primer ends of the target DNA and thus can be used to quantify the target DNA amplified by these primers. The competitor DNA fragments differ in size from the corresponding target DNA and are readily distinguished from them by agarose gel electrophoresis. After electrophoresis, fragments of appropriate size were excised and subcloned for sequencing as described above to verify that the competitor DNA did not overlap with the PCR products amplified from the targets. Differing amounts of competitor DNA were added to each sample prior to their amplification (Table 3). For quantitative analysis, the fluorescence of the agarose gels was photographed, scanned, and digitized. The optical density of the digitized images was analyzed by using Kodak Digital Science 1D Image Analysis v.1.51 software (Eastman Kodak Company, Rochester, NY) with a Macintosh computer.

In situ hybridization

A digoxigenin-labeled cRNA probe was prepared from mouse brain cDNA encoding a segment (nucleotides 2601–3952) of the sequence of DCC. The cloned plasmid DNA was linearized with either *Not*I or *Bam*HI, purified, and T7 and SP6 RNA polymerases were used to transcribe, respectively, anti-sense and sense cRNA probes. Digoxigenin-labeled cRNA probes for mouse netrin-1, chick netrin-2, and rat DCC were prepared, respectively, from plasmids pMNET-3′ UTR, pCM25, and D-24 (supplied by M. Tessier-Lavigne). The riboprobes were subjected to alkaline hydrolysis before precipitation to reduce their sizes to <200 bp in order to improve tissue penetration (Schaeren-Wiemers and Gerfin-Moser, 1993). The riboprobes were quantified by dot blotting by using a commercially supplied protocol (DIG-RNA labeling kit; Roche Molecular Biochemicals, Indianapolis, IN). In situ hybridization (Conlon and Rossant, 1992) was carried out by incubating sections with sense (control) or anti-sense probes (0.2–0.5 ng/μl; in 100-μl wells) in a moist chamber at 60°C for 16 h. The hybridization buffer contained 50% formamide, 5× saline sodium citrate buffer (SSC; pH 4.5), 50 μg/ml yeast

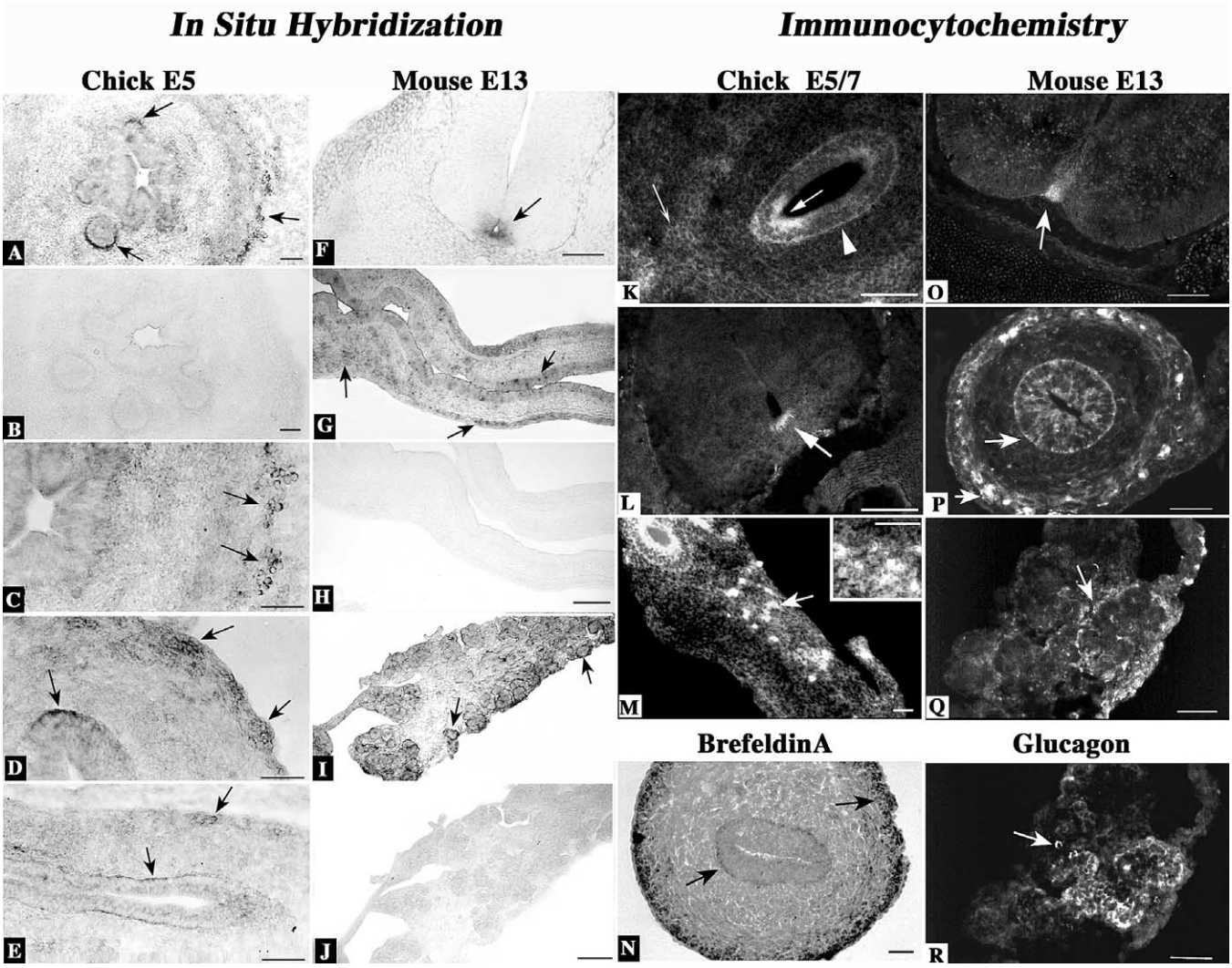


Fig. 2. The cellular locations of netrin transcripts and immunoreactivity were analyzed in the developing gut and pancreas by in situ hybridization and immunocytochemistry. (A) Chick proventriculus at E5. Antisense probe. Transcripts encoding netrin-2 are found in basal epithelial cells and in cells within the outer gut mesenchyme in the region of developing myenteric ganglia. (B) Chick proventriculus at E5. Sense probe (control). No cells are labeled. (C, D) Chick proventriculus (C) and gizzard (D) at E5. Antisense probe. At higher magnification, the labeled cells (arrows) of the outer gut mesenchyme are seen to form aggregates and the epithelial labeling (D) to be sharply restricted to the basal cytoplasm. (E) Chick duodenum at E5. Antisense probe. Transcripts encoding netrin-2 are found in developing myenteric ganglia and in the basal cytoplasm of epithelial cells. (F) Mouse spinal cord at E13 (examined as a positive control). Antisense probe. Transcripts encoding netrin-1 are located in the floor plate (arrow). (G) Mouse duodenum at E13. Antisense probe. Transcripts encoding netrin-1 are located in the outer gut mesenchyme (region of developing myenteric ganglia; arrows) and the mucosal epithelium. (H) Mouse duodenum at E13. Sense probe (control). No cells are labeled. (I) Mouse pancreas at E13. Antisense probe. Transcripts encoding netrin-1 are located in the basolateral cytoplasm of acinar cells (arrows). (J) Mouse pancreas at E13. Sense probe (control). No cells are labeled. (K) Chick small intestine at E7. Netrin immunoreactivity is found in mucosal epithelial cells and diffusely in the outer gut mesenchyme (arrows). Immunoreactivity appears to be concentrated in the region of the mucosal basement membrane (arrowhead). (L) Chick spinal cord at E7 (analyzed as a positive control). Immunoreactivity is confined to the floor plate. (M) Chick gut and pancreas at E5. Netrin-immunoreactive cells are present in the dorsal pancreatic bud (arrows) and inset. (N) Chick small intestine at E7 fixed after incubation for 4 hrs with brefeldin A. Netrin immunoreactivity (arrows) has accumulated in cells of the outer gut mesenchyme. (O) Mouse spinal cord at E13 (analyzed as a positive control). Netrin immunoreactivity is located in the floor plate (arrow). (P) Mouse small intestine at E13. Netrin immunoreactivity (arrows) is found in cells of the outer gut mesenchyme in the region where crest-derived cells are known to migrate and in the mucosal epithelium. (Q) Mouse pancreas at E13. Netrin immunoreactivity is concentrated in the basolateral cytoplasm of acinar cells. (R) The same section as in (Q) but the light is filtered to demonstrate the immunoreactivity of glucagon, a marker for developing islets of Langerhans. The immunoreactivities of netrin (Q) and glucagon (R) do not coincide. Scale bars: (A–E) 50 μm; (F–J) 100 μm; (K) 25 μm; (L) 50 μm; (M, N) 25 μm; (O–R) 50 μm.

tRNA, 1% sodium dodecyl sulfate (SDS), and 50 μg/ml heparin. Following hybridization, preparations were washed four times in 5× SSC (pH 4.5)/1% SDS/50% formamide at 60°C, and three times in 5× SSC (pH 4.5)/50% formamide at 55°C. Bound digoxigenin was detected with antibodies coupled to alkaline phosphatase (diluted 1:1500; Roche) or HRP (ABC method, Elite kit; Vector Laboratories). Alkaline phosphatase activity was demonstrated and visualized

with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche) in a buffer containing 100 mM NaCl, 50 mM $MgCl_2$, 1% Tween 20, and 100 mM Tris–HCl at pH 9.5. The endogenous alkaline phosphatase activity of the intestines was inhibited by incubating tissues with 250 μg/ml levamisole (Sigma).

Western blotting

The entire bowel from 15 chick embryos (E6) was homogenized in cold lysis buffer (Tris 0.02 M, Triton 0.1%, EDTA 1 mM) containing a commercial cocktail of protease inhibitors [diluted 1:10; a mixture of 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, and pepstatin A; Sigma]. When Western blots were used to analyze the effects of caspase and/or metalloprotease on DCC, the caspase inhibitors VI (100 μM) and III (25 μM) and metalloprotease inhibitor III (20 μM) (all from Calbiochem, San Diego, CA) were also added to the protein samples. The lysate was centrifuged at a low speed to remove debris, and the protein content of the supernatant was determined (Bio-Rad Laboratories, Richmond, CA). The supernatant was then boiled for 5 min at 95°C in Laemmli solution (Bio-Rad) containing 0.35 M dithiothreitol. An aliquot containing 20 μg of protein was subjected to SDS-PAGE (10% polyacrylamide). The separated proteins were electrophoretically blotted onto a nitrocellulose sheet for Western analysis. The blots were bleached for 5 min with 6% H_2O_2 and blocked by incubation for 2 h in TBST buffer (Tris 0.05 M, NaCl 0.15 M, Tween 0.05%) containing 5% fat-free milk. Immunoreactivity was identified with appropriate secondary antibodies (Table 2) conjugated to horseradish peroxidase (HRP; diluted 1:5000; Jackson Labs, West Grove, PA). HRP activity was visualized with 4-chloro-1-naphthol (4-CN Kit; Bio-Rad).

Results

Netrins and their receptors are expressed in the developing bowel and pancreas of chicks and mice

Transcripts encoding netrins are present in developing gut and pancreas

Initial experiments employed RT-PCR to determine whether transcripts encoding one or more netrins could be detected in the developing bowel and pancreas of chicks (E5-E19) and mice (E11-P14). The E11 chick brain was studied as a positive control. Transcripts encoding netrin-2 were found in the chick gut at the earliest age examined, E5, and continued to be present through E19 (Fig. 1A). Transcripts encoding netrin-1 were not detected in the bowel but were found in the E11 brain, suggesting that if netrin-1 is expressed in the chick gut at all, its level of expression must be extremely low. Competitive PCR revealed that netrin-2 expression in the chick bowel is developmentally regulated (Fig. 1D). Expression was maximal as a function of age at E12 and declined at E19 to $<$20% maximal. As in the chick gut, transcripts encoding netrin-1 were found at E7 and E13 in the chick pancreas (Fig. 1B), but those encoding netrin-1 were not detected at either age. Transcripts encoding two netrins, netrin-1 and netrin-3, were detected in both the fetal (E11–E15) and neonatal (P0–P14) murine bowel (Fig. 1C).

Sites of netrin expression in the enteric viscera of developing chicks (E5) and mice (E13) were located by in situ hybridization. The floor plate of the developing murine spinal cord (E13) was analyzed as a positive control. mRNA encoding netrin-2 was found to be expressed in the mesenchyme of the outer bowel and in the basal regions of epithelial cells in the chick preventriculus (Fig. 2A–C), gizzard (Fig. 2D), and duodenum (Fig. 2E). No labeling was observed when sections of chick gut were hybridized with a sense riboprobe (Fig. 2B). mRNA encoding netrin-1 was found in the floor plate of the murine spinal cord (Fig. 2F), and its distribution in the fetal mouse intestine, in the outer gut mesenchyme and epithelium, was similar to that in the bowel of chicks (Fig. 2G and H). mRNA encoding netrin-1 was also found in the primordial mouse pancreas, where it was prominent in the basal cytoplasm of acinar cells (Fig. 2I and J). No labeling was observed when sections of mouse gut or pancreas were hybridized with a sense riboprobe (Fig. 2H and J).

Netrin immunoreactivity is present in the developing gut and pancreas

Netrin immunoreactivity was located in the chick gut, pancreas, and spinal cord, which was processed as a positive control. Netrin immunoreactivity was detected in the bowel and pancreas at E5–E10 and in the spinal cord at E7. In the gut, netrin immunoreactivity was found in a diffuse pattern that outlined cells in the region of the presumptive circular

Fig. 3. DCC, neogenin, and the adenosine A2b receptor are expressed in the developing gut and pancreas of chicks and mice. (A) mRNAs encoding DCC and neogenin can be detected in the developing mouse gut (E11–P14) by RT-PCR. (B) mRNA encoding neogenin can be detected in the developing chick gut (E4–E19). fg, foregut; hg, hindgut; wg, whole gut; br, brain. (C) Immunoblots probed with antibodies to DCC in the absence (lane 1) or presence (lane 2) of inhibitors of caspases and metalloproteases. The protein band at 150 kDa (arrowhead), the expected size of the full-length DCC, is more intense when protein was extracted in the presence of inhibitors of caspases and metalloproteases. (D) Expression of mRNA encoding DCC analyzed with competitive PCR in the developing mouse gut. DCC expression is developmentally regulated. Note that the level of DCC expression in the postnatal gut is quite low relative to that in the fetal bowel (maximal E13-E15). (E) Expression of mRNA encoding neogenin analyzed with competitive PCR in the developing chick gut. Neogenin expression gradually declines as a function of age. (F) Expression of mRNA encoding the adenosine A2b receptor analyzed with competitive PCR in the developing chick gut. Adenosine A2b receptor expression declines as a function of age. (G) Competitive PCR. mRNAs encoding neogenin and the adenosine A2b receptor can be detected in the chick pancreas at E7.5 and E13.

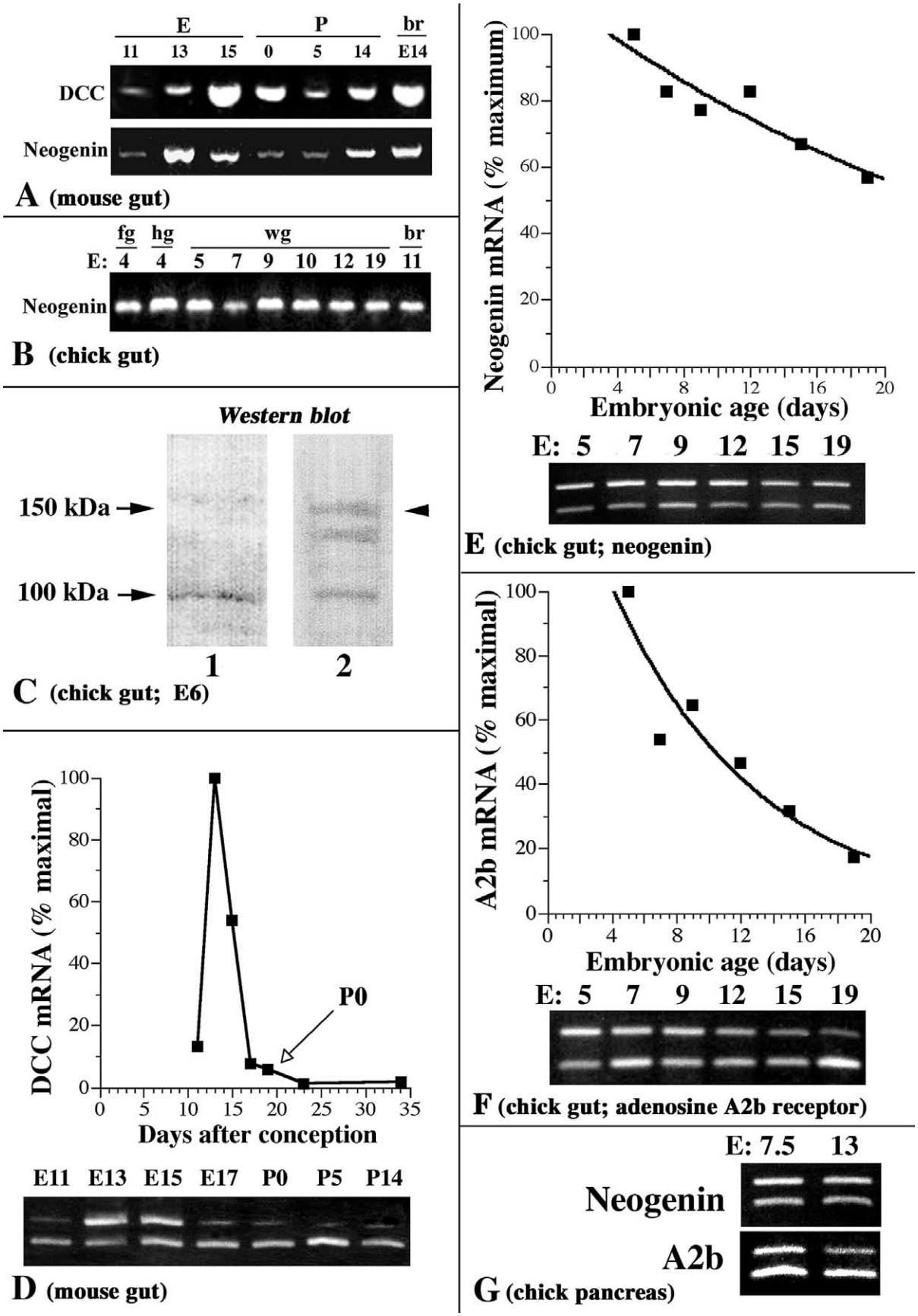

E
P
br
11
13
15
0
5
14
E14
DCC
Neogenin
A (mouse gut)
fg
hg
wg
br
E: 4
4
5
7
9
10
12
19
11
Neogenin
B (chick gut)
Western blot
150 kDa
100 kDa
1
2
C (chick gut; E6)
DCC mRNA (% maximal)
P0
Days after conception
E11
E13
E15
E17
P0
P5
P14
D (mouse gut)
Neogenin mRNA (% maximum)
Embryonic age (days)
E: 5
7
9
12
15
19
E (chick gut; neogenin)
A2b mRNA (% maximal)
Embryonic age (days)
E: 5
7
9
12
15
19
F (chick gut; adenosine A2b receptor)
E: 7.5
13
Neogenin
A2b
G (chick pancreas)

Fig. 3

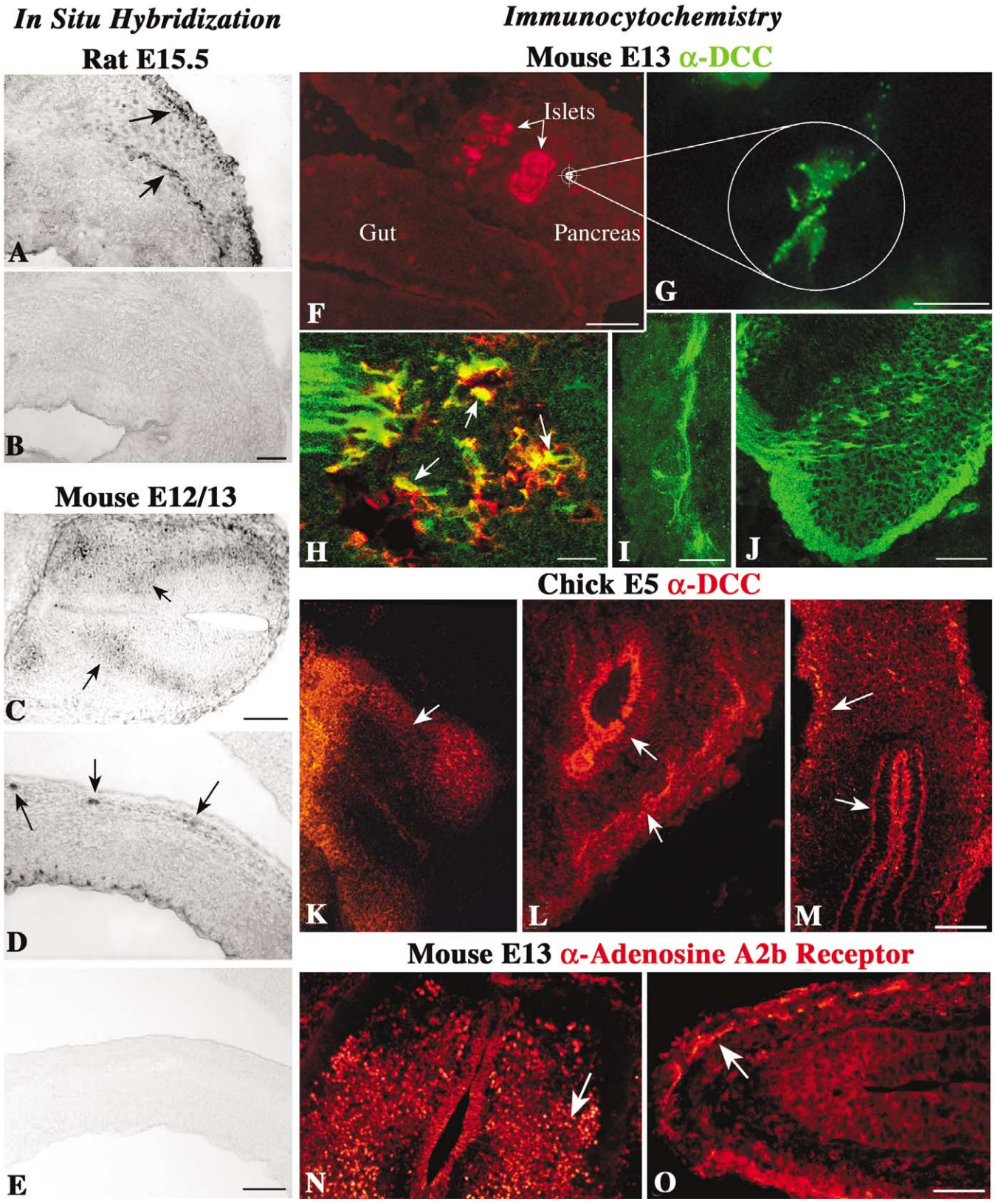

In Situ Hybridization
Rat E15.5
A
B
Mouse E12/13
C
D
E
Immunocytochemistry
Mouse E13 α-DCC
Islets
Gut
Pancreas
F
G
H
I
J
Chick E5 α-DCC
K
L
M
Mouse E13 α-Adenosine A2b Receptor
N
O

Fig. 4

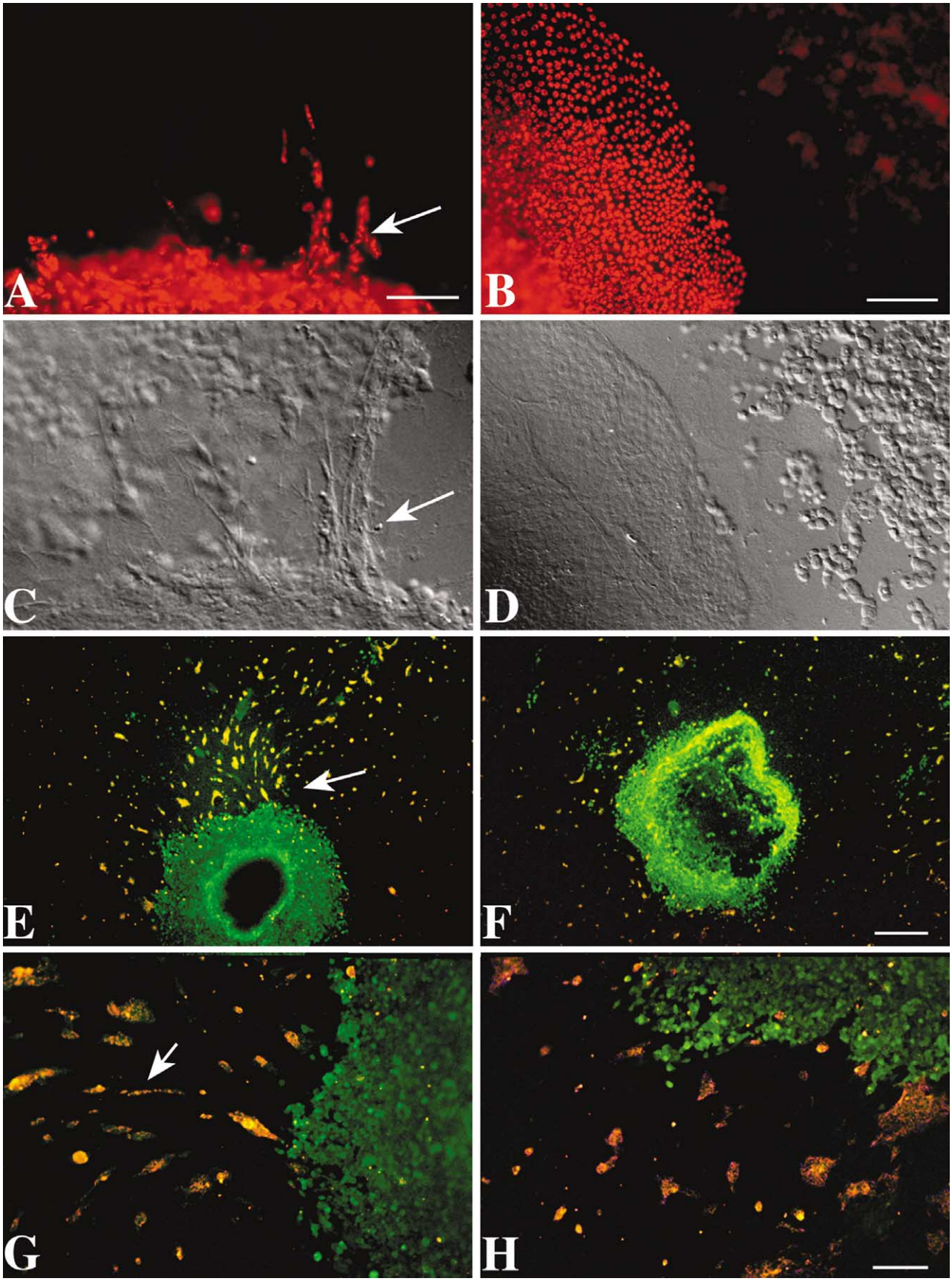


Fig. 5

muscle and in the mucosal epithelium and its underlying basement membrane (Fig. 2K and M). In the spinal cord, netrin immunoreactivity was restricted to the floor plate (Fig. 2L). Netrin immunoreactivity was also detected in cells within the mesenchyme of the developing dorsal pancreatic bud as early as E5 (Fig. 2M). Since the cells that secrete netrins may do so constitutively and not store them intracellularly, these cells may be difficult to demonstrate by immunocytochemistry. To identify cells that synthesize netrins, segments of gut were thus incubated with brefeldin A, which causes the Golgi apparatus and endoplasmic reticulum to fuse into a single compartment, within which even constitutively secreted products accumulate (Klausner et al., 1992). Following incubation with brefeldin A, cells were no longer surrounded by netrin-immunoreactivity, which instead was now found within cells of the outer gut mesenchyme and in the basal cytoplasm of mucosal epithelial cells (Fig. 2N). These are likely to be the cells of the chick gut in which netrin-2 is synthesized. The congruent localization of netrin immunoreactivity after brefeldin A (Fig. 2N) and that of transcripts encoding netrin-2 (Fig. 2K and M) supports the idea that these are the sites of netrin expression in the developing chick bowel.

The locations of sites of netrin immunoreactivity in the developing (E13) mouse spinal cord (Fig. 2O), gut (Fig. 2P), and pancreas (Fig. 2Q) were similar to those of the chick. Netrin immunoreactivity displayed a more cellular pattern of localization in the outer gut mesenchyme than in the chick; the cells, moreover, were located in the region of the presumptive myenteric plexus (Fig. 2P). Again, considerable immunoreactivity was found at the base of the mucosal epithelium. The pancreatic netrin immunoreactivity was concentrated around the basolateral borders of acinar cells (Fig. 2Q). The sites of netrin immunoreactivity were not coincident with those of glucagon, which was immunostained in the same sections to locate developing islets of Langerhans (Fig. 2R, compare with Fig. 2Q). The netrin immunoreactivity was most intense, however, near developing islet cells.

Netrin receptors are expressed in the developing gut and pancreas of chicks and mice

Expression of DCC and neogenin was analyzed in the gut of fetal mice by using RT-PCR. The brain was also studied as a positive control. Transcripts encoding DCC and neogenin were detected at E11, the earliest age examined, and they continued to be expressed in the gut through P14 (Fig. 3A). Transcripts encoding both DCC and neogenin were also found in the E14 mouse brain (Fig. 3A). DCC has not yet been sequenced in chicks; therefore, in the chick, the expression of transcripts encoding neogenin, but not of encoding DCC was investigated with RT-PCR (Fig. 3B). Transcripts encoding neogenin were detected in both the foregut and hindgut of chicks at E4, the earliest age examined, and continued to be present through E19 (Fig. 3B). Transcripts encoding neogenin were also found in the E11 chick brain (Fig. 3B).

The chick gut contains protein that reacts in immunoblots with antibodies to human DCC (Fig. 3C). A major band (~100 kDa) and a weaker band (~150–160 kDa), were seen (Fig. 3C, lane 1). The DCC gene encodes a group of structurally similar proteins of molecular mass ~150–190 kDa (Fazeli et al., 1997; Hedrick et al., 1994; Pierceall et al., 1994). DCC, however, is cleaved by caspases 20 and 40 kDa from its C-terminal end (Mehlen et al., 1998), and its ectodomain is shed from cells through the actions of

Fig. 4. The cellular locations of DCC transcripts and immunoreactivity were analyzed in the developing gut rats, mice, and chicks by in situ hybridization and immunocytochemistry. (A) Rat duodenum at E15.5. Antisense probe. Label is present in the myenteric plexus (upper arrow) and in the primordial submucosal plexus (lower arrow). (B) Rat duodenum at E15.5. Sense probe. No cells are labeled. (C) Mouse spinal cord at E12 (positive control). Antisense probe. Transcripts encoding DCC are found in commissural neurons and motor columns. (D) Mouse small intestine at E13. Antisense probe. Transcripts encoding DCC are located in cells external to the developing circular muscle where myenteric ganglia form (arrows) and in some mucosal epithelial cells. (E) Mouse small intestine at E13. Sense probe. No cells are labeled. DCC immunoreactivity is expressed in the developing gut and pancreas of E13 mice and E5 chicks. (F) Mouse gut and pancreas at E13 immunostained with antibodies to glucagon to identify the presumptive pancreas and to locate developing islets of Langerhans. The dorsal pancreatic bud is adjacent to the fetal gut. The marker (ϕ) indicates the location of the DCC-immunoreactive cells that are illustrated in the field outlined by the circle in (G). (G) DCC-immunoreactive cells in the E13 mouse pancreas found at the location indicated by the (ϕ) in (F). (H) Confocal image. Double label immunocytochemical demonstration of DCC (green) and the neural marker PGP 9.5 (red) in the region of the primordial myenteric plexus of the E13 gastric antrum. Coincident labeling is found in many cells (yellow; arrows), while others express only DCC or PGP9.5 immunoreactivities. A dense bundle of DCC-immunoreactive nerve fibers enters the field at the left. (I) Confocal image. DCC-immunoreactive cells in the primordial myenteric plexus of the E13 mouse gut. (J) Confocal image. DCC immunoreactivity in the E13 mouse spinal cord (positive control). (K) Chick spinal cord (positive control). Neurons exhibit DCC immunoreactivity (arrow). (L, M) Chick gut E5. DCC immunoreactivity is present in the myenteric plexus and in mucosal epithelial cells (arrows). (N) Mouse spinal cord at E13. Adenosine A2b receptor immunoreactivity is present in many cells (arrow). (O) Mouse small intestine at E13. The myenteric plexus (arrow) contains adenosine A2b receptor immunoreactivity. Scale bars: (A, B) 50 μm; (C–E) 100 μm; (F) 100 μm; (G–I) 25 μm; (J–H) 100 μm; (N, O) 50 μm.

Fig. 5. Enteric crest-derived cells migrate toward co-cultured sources of netrin-1. (A, C) An explant of E5 quail gut was cocultured with netrin-I-expressing HEK293 cells. Quail cells were identified by immunoreactivity with QCPN antibodies (A, Cy3, red fluorescence). The position of the grafts was determined by interference contrast microscopy. Quail cells migrate out of the gut toward the cocultured netrin-expressing cells. (B, D) Quail gut was cocultured with control nontransfected HEK293 cells. QCPN-immunoreactive cells do not leave the explants of gut (B) and do not migrate toward the cocultured HEK293 cells (D). (E–H) Crest-derived cells were immunoselected from the E6 chick gut with HNK-1 antibodies. The isolated enteric crest-derived cells were cocultured with three-dimensional collagen gels containing HEK293 cells that either expressed netrin-1 (E, G) or were nontransfected controls (F, H). Note that HNK-1-immunoreactive crest-derived cells (arrow, Cy3 red fluorescence) became oriented toward the netrin-1-expressing, but not the control HEK293 cells (PGP9.5-immunoreactive, FITC green fluorescence). Scale bars: (A, C) 50 μm; (B, D) 100 μm; (E, F) 500 μm; (G, H) 125 μm.

metalloproteases of the ADAM class (Galko and Tessier-Lavigne, 2000). Immunoblotting was thus repeated by using caspase inhibitor VI and MMP inhibitor III. When this was done, major bands became apparent in the immunoblots at ~130 and ~150 kDa, while the relative intensity of the ~100-kDa band decreased (Fig. 3C, lane 2). These data are consistent with the ideas that chick DCC migrates at ~160 kDa, that it is a substrate for caspases and metalloproteases, that the ectodomain, which remains after caspase and/or metalloproteases cleavage, is ~100 kDa, and that chick DCC is recognized by antibodies to human DCC.

In the bowel of the E11 mouse, the level of expression of DCC was found by competitive PCR to be low (Fig. 3D). The expression of DCC increased sharply on day E13, declined moderately by E15, and then more rapidly by E17. By the time of birth, the level of DCC expression was less than that at E11 and declined still further to reach a very low level of expression that was maintained through P14 (Fig. 3D). The level of expression of neogenin was relatively high early in the development of the chick gut, at E5, but then gradually declined through E19 (Fig. 3E). The expression of the adenosine A2b receptor was examined in the developing chick gut, even though its role in netrin signaling is not clearly established. The adenosine A2b receptor was found to be expressed in the chick bowel during early development, at E15, but its expression declined as a function of age (Fig. 3F) even more sharply than did that of neogenin. Transcripts encoding neogenin and the adenosine A2b receptor were also observed in the chick pancreas; the level of expression of neogenin in the pancreas did not change between E7.5 and E13, but that of the adenosine A2b receptor declined (Fig. 3G).

In situ hybridization was used to locate sites of expression of DCC in the developing bowel of rats (E15.5) and mice (E13). The expression of mRNA encoding DCC in the E12 mouse spinal cord, where it is known to be expressed by commissural neurons and the developing motor columns (Keino-Masu et al., 1996), was used as a positive control. In the E15.5 rat, mRNA encoding DCC was observed in the outer mesenchyme, in the region of the primordial myenteric and submucosal plexuses (Fig. 4A). In the mouse, strong labeling of developing cells was found in developing myenteric ganglia (Fig. 4D). The antisense probe also revealed the mRNA encoding DCC, as expected, in commissural neurons and the developing motor columns of the E12 mouse spinal cord (Fig. 4C). No labeling was detected when adjacent sections were hybridized with a sense riboprobe (Fig. 4B and E).

DCC and adenosine A2b immunoreactivities are expressed in the bowel by cells in a neural lineage

The immunoreactivities of DCC and the adenosine A2b receptor were located in the gut and pancreas of the E13 mouse and in the gut and spinal cord (which was processed as a positive control) of the E7 chick. Glucagon immunoreactivity was used to confirm the identification of the developing mouse pancreas and to locate primordial islets of Langerhans (Fig. 4F). DCC immunoreactivity was found to be located in scattered cells within the pancreas (Fig. 4G), where they were close to but not coincident with the islets (see the marker in Fig. 4F). Vagal nerve fibers descending in the wall of the stomach were found in a thick DCC-immunoreactive nerve bundle (Fig. 4H). These fibers terminated within the presumptive myenteric plexus where DCC-immunoreactive cells were found (Fig. 4H and I). A subset of these DCC-immunoreactive cells displayed coincident labeling with the immunoreactivity of the neuronal marker, PGP9.5, and the doubly labeled cells, in turn, were a subset of the PGP9.5-immunoreactive population. Some, but not all of the DCC-immunoreactive cells, therefore, have already developed as neurons, and some but not all of the developing enteric neurons at E13 contain DCC. Bundles of DCC-immunoreactive neurites were also observed in the primordial myenteric plexus of the presumptive stomach and duodenum (Fig. 4I). Many axons, including commissural axons were also DCC-immunoreactive in the E13 mouse spinal cord (Fig. 4J), which was examined as a positive control. DCC immunoreactivity was also located in the E5 spinal cord (Fig. 4K) and gut (Fig. 4L and M) of chicks. Cold acetone fixation was found to be necessary to demonstrate immunoreactivity in chick tissue, possibly because the antibodies (the same as used for the immunoblots described above) were directed against a mammalian protein. Immunoreactivity was found in the spinal cord in a pattern that was consistent with the immunostaining of dorsal and ventral commissural neurons (Fig. 4K). In the chick bowel, DCC immunoreactivity was seen in the developing myenteric plexus and the epithelium mucosa (Fig. 4L and M). The basal surface of mucosal epithelial cells was distinctly immunostained in the chick intestine (Fig. 4M). Adenosine A2b receptor immunoreactivity was found in neurons of the E13 mouse spinal cord (Fig. 4N) (Puffinbarger et al., 1995; Stehle et al., 1992) and the myenteric plexus of the small intestine (Fig. 4O).

Netrin-1 attracts migrating enteric crest-derived cells by affecting DCC

Netrin-1 attracts enteric crest-derived cells

Explants of quail small intestine were cocultured with stably transfected HEK 293 cells that expressed netrin-1. The explants were embedded in a collagen gel to allow gradients to form and to prevent the dispersal of the HEK 293 cells. Control explants were cocultured with parent nontransfected HEK 293 cells. Cells from the explants of gut were identified immunocytochemically with QCPN antibodies (Table 1), while the HEK 293 cells were identified by interference contrast microscopy. Quail cells were found to migrate out of the explants of intestine and grow toward netrin-1-expressing (Fig. 5A and C), but not control (Fig. 5B and D) HEK 293 cells. The quail cells did not leave

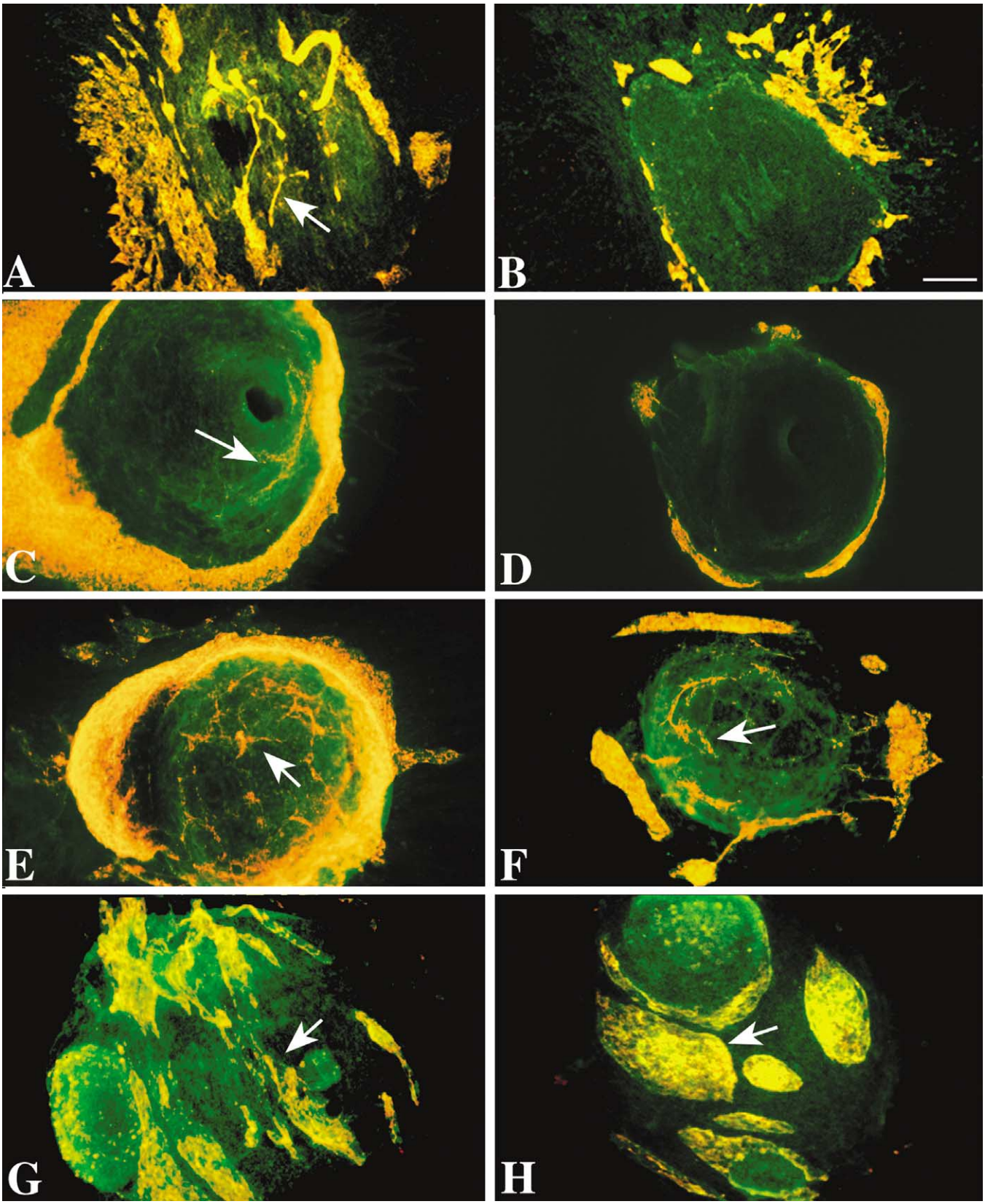


Fig. 6. Crest-derived cells migrate inwardly by a DCC-dependent mechanism in cultured rings of bowel. E5 chick gut was cut transversely and grown in organotypic tissue culture. (A) Control, no additives. HNK-1 immunoreactive crest-derived cells (arrow) migrate in toward the center of the cultures. (B) Antibodies to DCC. The inward migration of crest-derived cells is prevented and they remain frozen in their original locations at the periphery of the bowel. (C) SP-cAMPS. Crest-derived cells (arrow) continue to migrate inwardly. (D) RP-cAMPS. Crest-derived cells fail to migrate inwardly. (E) Antibodies to HNK-1. Crest-derived cells (arrow) continue to migrate inwardly. (F) Antibodies to PGP9.5. Crest-derived cells (arrow) continue to migrate inwardly. (G, H) The absence (G) or presence (H) of antibodies to the adenosine A2b receptor does not detectably affect the inward migration of crest-derived cells. Scale bars: (A–H) 100 μm.

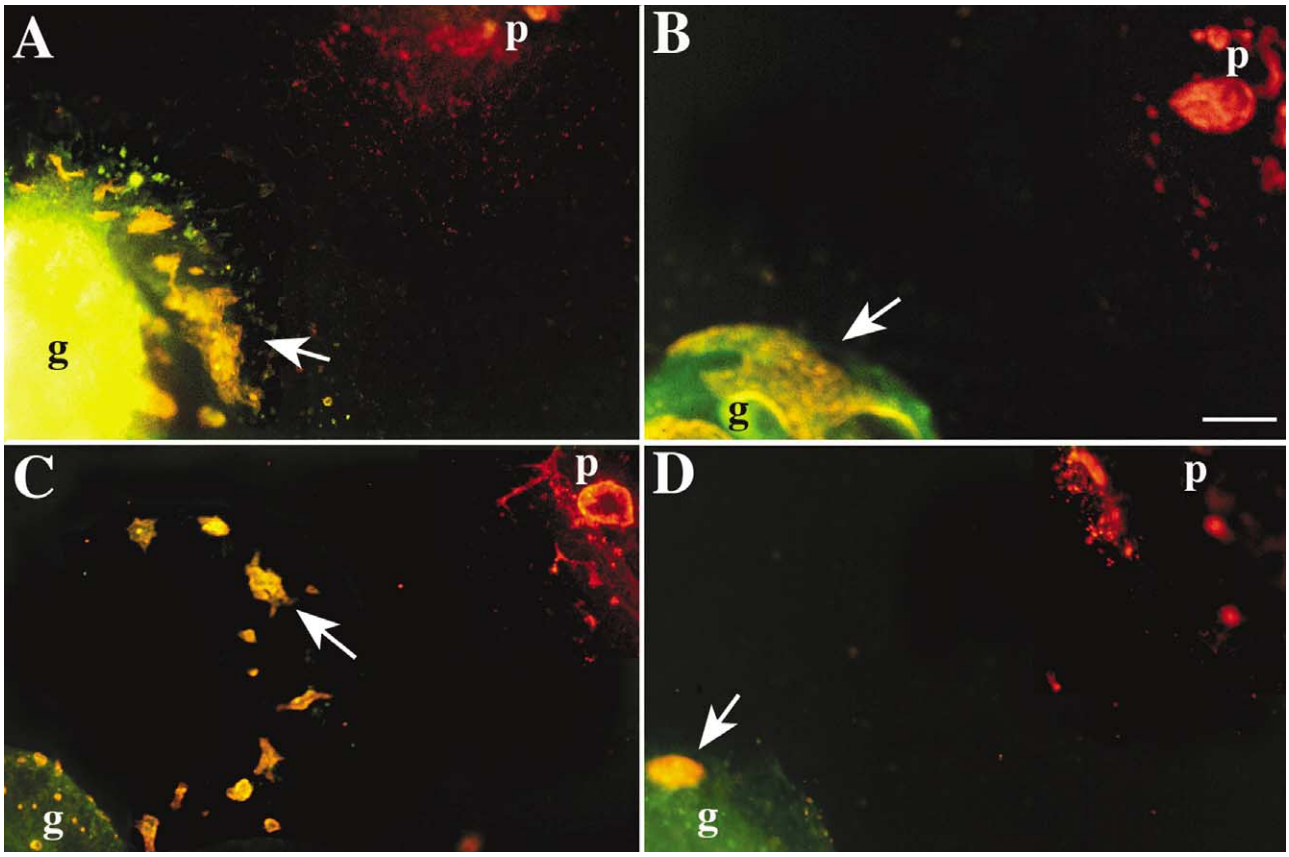


Fig. 7. Crest-derived cells migrate out of the gut toward co-cultured explants of pancreas; this migration is DCC-dependent. E5 chick gut was preincubated with CellTracker Green and then grown for 2 days in organotypic tissue culture with an explant of dorsal pancreatic bud. Crest-derived cells were identified by their HNK-1 immunoreactivity (Cy3, red fluorescence). (A) Control, no additives. Doubly labeled (CellTracker Green/HNK-1 immunoreactive) crest-derived cells (arrow) from the gut (g) migrate toward the nonfluorescent pancreatic bud (p). (B) Antibodies to DCC. Doubly labeled crest-derived cells (arrow) remain in the gut (g) and do not migrate toward the pancreatic bud (p). (C) SP-cAMPS. Doubly labeled crest-derived cells (arrow) from the gut (g) migrate toward the pancreatic bud (p). (D) RP-cAMPS. Doubly labeled crest-derived cells (arrow) remain in the gut (g) and do not migrate toward the pancreatic bud (p). Scale bars: (A–D) 100 μm.

regions of the explants of gut that were not apposed to the netrin-1-expressing cells.

In a second set of experiments, crest-derived cells were immunoselected from the E6 chick gut (Pomeranz et al., 1993) and layered over HEK 293 cells that were embedded in a collagen gel. The crest-derived cells of the chick were visualized immunocytochemically with HNK-1 monoclonal antibodies. The HEK 293 cells were identified with antibodies to PGP9.5, which react with HEK 293 cells, but not with chick crest cells. The crest-derived cells migrated toward the HEK 293 cells when the HEK 293 cells were netrin-1-secreting (Fig. 5E and G); however, there was no detectable directional migration of crest-derived cells when they were plated over control HEK 293 cells (Fig. 5F and H).

Antibodies to DCC block the inward migration of crest-derived cell in intestinal explants

The E5 chick gut was explanted and cut transversely so that it could be grown in a ring configuration in organotypic tissue culture. When this was done, the crest-derived cells, which were originally in the outer gut mesenchyme (Gershon et al., 1993; McKeown et al., 2001; Payette et al., 1984) (see Fig. 4L), migrated inward toward the mucosa (Fig. 6A). HNK-1 immunoreactivity was used as a marker for crest-derived cells. The inward migration of crest-derived cells was blocked by antibodies to DCC, which froze the cells in their positions at the periphery of the ring of bowel (Fig. 6B). The response to DCC stimulation is mediated by cAMP and PKA (Ming et al., 1997); therefore, the response of cells to netrins can be inhibited by the PKA antagonist, Rp-cAMPS, but not by the agonist, Sp-cAMPS. Exposure of cultures to Sp-cAMPS did not prevent the inward migration of crest-derived cells in ring cultures of gut (Fig. 6C); however, Rp-cAMPS abolished this migration, as did the antibodies to DCC (Fig. 6D). The addition of control antibodies, HNK-1 (Fig. 6E) or PGP9.5 (Fig. 6F), to the ring cultures was without effect on the inward migration of crest-derived cells. Exposure of the ring cultures to antibodies to the adenosine A2b receptor also failed to prevent the inward migration of crest-derived cells (Fig. 6G and H). These experiments suggest that a DCC-dependent mecha-

nism attracts crest-derived cells that are migrating proximodistally in the outer gut mesenchyme to turn in the perpendicular direction and migrate toward the mucosa. The data do not support the idea that the adenosine A2b receptor is a necessary coreceptor for this function of DCC. The mucosa, which expresses netrins, might be the source of endogenous agonist to stimulate DCC on crest-derived cells. The inward migration of a subset of crest-derived cells could be important in the formation of the submucosal plexus of the small bowel.

Antibodies to DCC inhibit the migration of crest-derived cells from gut toward pancreas

Explants of E5 chick foregut were labeled by incubation with CellTracker Green and then cocultured for 2 days with dorsal pancreatic buds explanted at the same age. HNK-1 immunoreactivity was used to mark crest-derived cells; therefore, those crest-derived cells that are of enteric origin would be doubly labeled by CellTracker Green and HNK-1, while crest-derived cells that were in the pancreatic buds at the time of explantation, if any, would only be HNK-1-immunoreactive. Doubly labeled crest-derived cells were found to migrate away from the explants of bowel in the direction of the cocultured pancreatic bud (Fig. 7A). Few such cells migrated out of the enteric explants from surfaces that were not apposed to the pancreatic buds. When antibodies to DCC were included in the culture medium, no crest-derived cells migrated out of the explants of bowel (Fig. 7B). The exposure of cultures to Sp-cAMPS did not prevent the migration of doubly labeled crest-derived cells out the bowel in the direction of the pancreatic buds (Fig. 7C); however, this migration was blocked by Rp-cAMPS (Fig. 7D). These observations are consistent with the idea that the migration of crest-derived cells from the foregut to the pancreas (Kirchgessner et al., 1992) is a DCC-dependent response to the secretion of an attractant, probably a netrin.

Pancreatic and submucosal ganglia are absent in transgenic mice that lack DCC

Transgenic mice that lack DCC were investigated to test the hypotheses that chemoattractants secreted by the mucosa and pancreas induce DCC-expressing crest-derived cells in the small intestine to deviate from their proximodistal path of migration to enter, respectively, the submucosa and the pancreas. Antibodies to PGP9.5 were used as a marker for cells developing in a neural lineage. AChE activity was also demonstrated histochemically in some preparations to confirm the identification of pancreatic ganglia. At E12.5, the myenteric plexus of the wild-type gut is highly PGP9.5-immunoreactive and the primordial pancreas contains numerous PGP9.5-immunoreactive cells that have begun to form small ganglia (Fig. 8A and C) In contrast, the pancreas at the same age contains no PGP9.5-immunoreactive neurons (Fig. 8B and D), although the PGP9.5 immunoreactivity of the myenteric plexus of the $DCC^{-/-}$ mice resembles that of wild-type animals (not illustrated). At E15.5, a thin ring of submucosal plexus can be recognized as beginning to form in wild-type (Fig. 8E), but not in $DCC^{-/-}$ animals (Fig. 8F and H). Pancreatic neurons are also present in wild-type (Fig. 8E and G) but not in $DCC^{-/-}$ mice (Fig. 8F and H). At P0, pancreatic neurons could again be recognized in the pancreas of wild-type animals (Fig. 8I and K), but they were still absent from the pancreas of $DCC^{-/-}$ mice (Fig. 8J and L). By this age the close relationship of pancreatic ganglia to Islets of Langerhans was evident in wild-type mice (Fig. 8K). Evidence of the formation of a submucosal plexus was still not apparent in the P0 $DCC^{-/-}$ animals (Fig. 8L). The small pancreatic ganglia of wild-type P0 mice displayed typical AChE activity (Fig. 8M); no AChE-stained ganglia were evident at P0 in $DCC^{-/-}$ mice (Fig. 8N). These observations are consistent with the idea that netrins secreted by the intestinal mucosa and the pancreas play roles, respectively, in the formation of the submucosal plexus and pancreatic ganglia.

Netrin-1 promotes the development and/or survival of enteric crest-derived cells

DCC is thought to induce apoptosis when it is deprived of ligand binding, but to promote survival when it is engaged by netrin-1 (Forcet et al., 2001; Mehlen et al., 1998). If a subset of crest-derived cells expresses functional DCC, therefore, then their development/survival would be expected to be promoted by netrin-1. This hypothesis was tested. The E7 chick gut was dissociated and the resulting cell suspension was either plated directly on collagen-coated coverslips for culture or subjected to immunoselection with HNK-1 antibodies to isolate crest-derived cells. Cultures of mixed cells from the dissociated bowel or isolated crest-derived cells were then exposed to netrin-1 (0.02-1.0 μg/ml). Netrin-1 was found to increase the number of crest-derived cells, identified immunocytochemically with HNK-1 antibodies, in both types of culture; however, the efficacy of netrin-1 was substantially greater for the cultures of immunoselected cells (Fig. 9A). The concentration-effect relationship for netrin-1 was bell-shaped; the maximal development/survival was reached at $\sim$0.5 μg/ml and declined at 1.0 μg/ml. At the optimum concentration of netrin-1, the mean number of surviving crest-derived cells was 171% of control ($P < 0.02$) in cultures of mixed cells and 261% of control ($P < 0.0001$) in cultures of immunoselected cells. Netrin-1 thus promotes the in vitro development/survival of enteric crest-derived cells. The effect of netrin-1 does not require the secretion of a cofactor produced by non-crest-derived cells. Exogenous netrin-1 probably exerts a greater effect in cultures of isolated crest-derived cells than in cultures of mixed cells from the gut wall because endogenous mesenchymal cells, which are included in the latter cultures, express netrin-1.

Netrin-1 promoted the outgrowth of neurites as well as the development/survival of enteric crest-derived cells;

however, netrin-1 was only able to do so in cultures of mixed cells of the gut wall. Process bearing cells (PBCs) were defined as those extending neurites >3-fold the maximal diameter of the perikaryon. In control cultures of mixed cells (Fig. 9B), PBCs constituted 9.3 ± 3.4% of the total number of HNK-1-immunoreactive crest-derived cells; netrin-1 (0.5 μg/ml) increased this proportion to 17.9 ± 1.7% ($P < 0.05$). Netrin-1 did not significantly increase this proportion in cultures of isolated crest-derived cells (Fig. 9B). These data suggest that the promotion of neurite extension by netrin-1 requires co-factor(s) produced by non-crest-derived cells of the bowel wall.

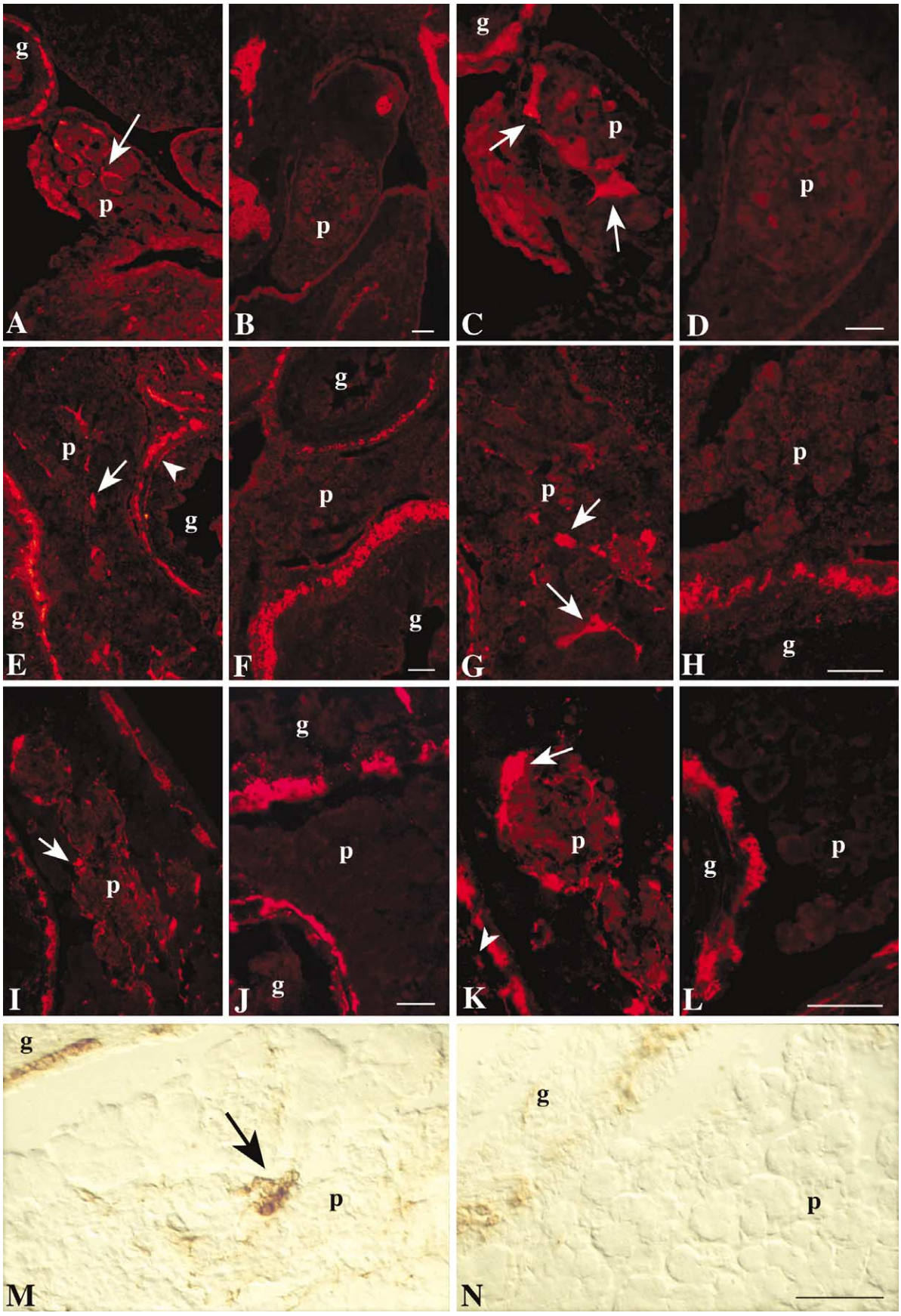
Discussion

The current study was undertaken to test the idea that netrins function as guidance molecules that attract enteric crest-derived cells to their correct destinations in the bowel and/or pancreas. Initial studies were designed to determine whether the elements of the netrin signaling system (netrins and their receptors) are present in the gut and/or pancreas at appropriate times during development. Transcripts encoding netrin-2 are expressed in the gut of chicks and those encoding netrins-1 and -3 are expressed in the bowel of mice. The same netrins are expressed respectively in the chick and mouse pancreas. Netrin expression, quantified in the chick gut by competitive PCR, is developmentally regulated, increasing during the time crest-derived cells are migrating, particularly when the submucosal plexus is formed (Allan and Newgreen, 1980; Burns and Le Douarin, 1998; McKeown et al., 2001; Payette et al., 1984), and decreasing afterward. Sites of mRNA encoding netrins in the bowel, located by in situ hybridization, and sites of netrin protein concentration, located by immunocytochemistry, are similar in mice and chicks; both mRNA and netrin immunoreactivity are found in the basal regions of mucosal epithelial cells and in the outer gut mesenchyme where netrin-1 RNA and netrin immunoreactivity were found in the region of developing myenteric ganglia. In the pancreas, mRNA and netrin immunoreactivity were located in the basal region of acinar cells. Brefeldin A caused netrin immunoreactivity to accumulate in the same sites in the chick gut where netrin mRNA was found by in situ hybridization, suggesting that netrins are constitutively secreted.

DCC expression, quantified in the murine gut by competitive PCR, was detectable as early as E11. Neurons are not recognizable at this age, but crest-derived cells are known to be present in the small intestine (McKeown et al., 2001; Rothman and Gershon, 1982). The hindgut is colonized later, at E12.5-E14.5 (McKeown et al., 2001; Rothman and Gershon, 1984); therefore by E13, when DCC expression reaches a peak, many crest-derived cells are migrating in the gut; furthermore, the period from E13 to E15 coincides with the secondary migrations of crest-derived cells in the small intestine from the outer gut mesenchyme to the submucosa and pancreas. The developmental regulation of DCC, therefore, is consistent with its expression by migrating enteric crest-derived cells. Neogenin and the adenosine A2b receptor are also expressed in the bowel; however, their expression slowly declines as a function of age. DCC was also found by in situ hybridization and immunocytochemistry to be expressed by developing enteric neurons; mRNA encoding DCC was located in myenteric ganglia and DCC immunoreactivity coincided in a subset of cells with that of the neural marker, PGP9.5. These data suggest that a subset of enteric crest-derived cells expresses DCC.

Several experiments confirmed that enteric crest-derived cells are attracted by netrin-1. Cells migrate out of explants of chick bowel toward cocultured masses of netrin secreting cells, but they do not migrate toward similarly cocultured cells that do not secrete netrin-1. Isolated enteric crest-derived cells also selectively migrate in vitro toward immobilized netrin-secreting cells. Experiments further suggested that enteric crest-derived cells migrate in vitro toward endogenous sources of netrins within the gut; thus, crest-derived cells migrate toward the mucosa in explanted rings of bowel. This inward migration was blocked by antibodies to DCC and by the PKA inhibitor, Rp-cAMPS, but not by antibodies to HNK-1 or PGP9.5, or by Sp-cAMPS (which does not inhibit PKA), suggesting that the migration requires DCC signal transduction. The inward migration of crest-derived cells was not affected by antibodies to the adenosine A2b receptor, an observation that is consistent with the report that adenosine A2b is not an essential coreceptor for DCC (Stein et al., 2001). Similar results were obtained with cocultures of gut and pancreas. Crest-derived cells migrate from the presumptive duodenum toward explants of pancreas and this migration, like the inward migration of crest-derived cells in intestinal rings, was abolished by antibodies to DCC and by RP-cAMPS.

The in vitro data suggest that netrins attract enteric crest-derived cells and cause them to migrate by a DCC-dependent mechanism toward the mucosa or pancreas. Studies of mice lacking DCC suggest that endogenously secreted netrins probably act similarly and divert the migration of a subset of DCC-expressing crest derived cells toward the submucosa and pancreas; thus, no evidence of the formation of a submucosal plexus or pancreatic ganglia was observed in the DCC knockout animals. At E12.5 crest-derived cells are already present in the primordial pancreas of wild-type mice, although the submucosal plexus has not yet begun to form; however, as late as P0, the DCC-deficient mice still lack pancreatic ganglia. By E15.5, submucosal ganglia have begun to form in the small intestine of wild-type mice, but again, even as late as P0 submucosal ganglia were not seen in the DCC-deficient animals. The lack of DCC evidently does not prevent crest-derived cells from colonizing the gut. The bowel was encircled by PGP9.5-immunoreactive neurons in both wild-type and DCC-deficient mice. Observations are thus consistent with the hypotheses that a DCC-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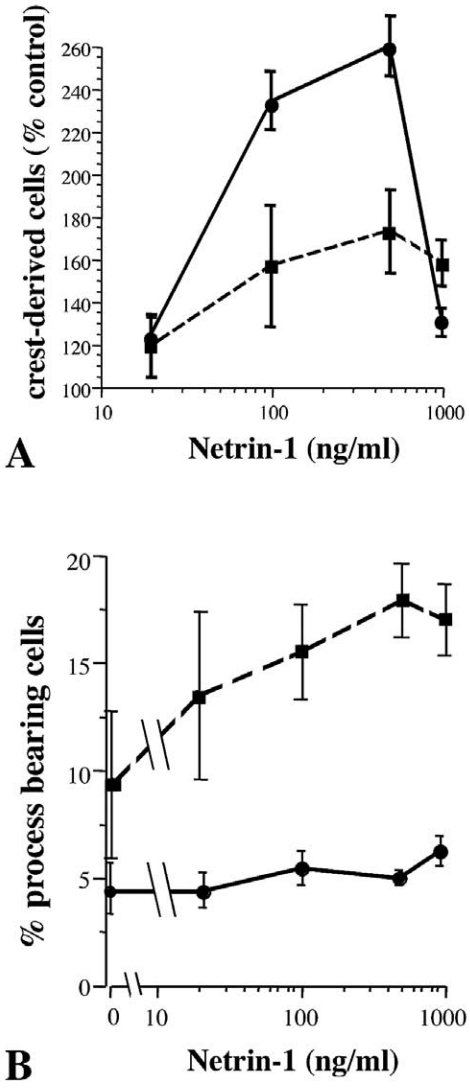

Fig. 9. Netrin-1 promotes the survival of enteric crest-derived cells in vitro. (A) Crest-derived cells were isolated from the E7 chick gut by immunoselection with antibodies to HNK-1 and cultured in the absence (control) or presence of ascending concentrations of netrin-1 (solid line). Alternatively, dissociated cells from the bowel were exposed to ascending concentrations of netrin-1 without prior immunoselection (dashed line). The number of surviving crest-derived cells (identified as HNK-1-immunoreactive) in each culture was determined, normalized as a percent of control (number of cells surviving in the absence of netrin-1), and the calculated means for isolated and mixed cells were plotted as a function of the netrin-1 concentration. The addition of netrin-1 promoted the survival/development of crest-derived cells in cultures of isolated cells to a greater extent than it did their survival in mixed cultures. The effect of netrin-1 was concentration-dependent but lost at a high concentration of the ligand. (B) The proportion of crest-derived cells that extended processes was quantified in cultures of isolated crest-derived (solid line) and mixed cells (dashed line) from the gut. Netrin-1 promoted the extension neurites (by HNK-1-immunoreactive cells) in cultures of mixed cells than to a greater extent than it did in cultures of isolated enteric crest-derived cells.

expressing subset of crest-derived cells migrating in the bowel attracted to netrins that are secreted by the mucosa; these cells migrate inwardly through the developing circular muscle and give rise to the submucosal plexus. The data are also consistent with the idea that another subset of crest-derived cells are attracted to netrins that are secreted by pancreatic acinar cells; these crest-derived cells migrate into pancreatic buds and give rise to pancreatic ganglia. The abilities of vagal crest-derived cells to "home" to the bowel and migrate proximodistally within it are probably independent of netrin/DCC signaling and thus occur in its absence.

The crest-derived cells that are putatively attracted by mucosal netrins do not actually reach the mucosa, although they often do so in vitro (see Fig. 6). Additional shorter range factor(s) may be secreted by the mucosa to act as stop or repulsive signals, which prevent crest-derived cells from migrating any further than the submucosa. One such factor may be the laminin that is secreted in abundance by the developing enteric mucosa (Chalazonitis et al., 1997b; Simon-Assmann et al., 1998). Laminin converts the attractive effects of netrin to repulsion (Hopker et al., 1999) and also promotes the development of enteric neurons (Chalazonitis et al., 1997b). Mucosally secreted laminin could thus repel approaching crest-derived cells, preventing them from entering the mucosa, while at the same time encouraging them to differentiate (and thus cease to migrate) in the submucosa. Alternatively, since Slit proteins and Robo receptors are expressed in the fetal gut (J. Chen and M.D.G., unpublished observations) and Robo downregulates DCC expression (Stein and Tessier-Lavigne, 2001), the failure of crest-derived cells to enter the mucosa could be due to the mucosal secretion of a short-range factor, such as Slit.

It is not entirely clear why, if mucosal netrin attracts crest-derived cells, only a relatively small subset migrates into the submucosa. DCC is expressed by only a subset of the PGP9.5-expressing enteric crest-derived cells (see Fig. 4H); therefore, the response of some cells and not others to the putative attraction of mucosal netrin might be explained if only the DCC-expressing subset of neural precursors were able respond to mucosal netrin. Timing or the expression of a coreceptor may also be important; conceivably, only a subset of the DCC-expressing cells may be capable of migrating when a netrin gradient from the mucosa reaches the outer gut mesenchyme, which itself is a source of netrin. The netrin in the outer gut mesenchyme may function in the attraction of vagal fibers, which were found to express DCC, to the developing myenteric ganglia.

The presence of netrin in the outer gut mesenchyme may

Fig. 8. Submucosal and pancreatic ganglia are not seen in $DCC^{-/-}$ mice. Sections showing the developing duodenum and bowel in wild-type and $DCC^{-/-}$ mice are compared at E12.5 (A–D), E15.5 (E–H), and PO (I–N). The sections from wild-type mice are in the vertical rows (A, E, I, M and C, G, K) while the $DCC^{-/-}$ mice are in the vertical rows (B, F, J and D, H, L, N). Neurons (arrows) are demonstrated immunocytochemically with antibodies to PGP9.5 (A–L) and by visualizing AChE activity (M, N). g, gut; p, pancreas. Note that primordial pancreatic ganglia with PGP9.5-immunoreactive cells can be discerned at all ages in wild type mice but not $DCC^{-/-}$ animals. A primordial submucosal plexus has begun to form in wild-type mice by E15.5 (E) and is seen also at PO (K) but is not present in the $DCC^{-/-}$ bowel at this age or even at PO (J, L). Scale bars: (A–H) 50 μm; (I–N) 100 μm.

be important in preventing premature apoptosis among the migrating crest-derived cells, because in the absence of ligand, DCC mediates apoptosis (Forcet et al., 2001; Llambi et al., 2001; Mehlen et al., 1998). The ability of netrins to prevent DCC-mediated apoptosis is likely to account for the promotion by netrin-1 that we observed in the development/survival of cultured crest-derived cells. No loss of myenteric cells was observed in DCC knockout mice. The knockout of DCC, however, would not be expected to increase cell death because the DCC-related apoptosis is actually DCC mediated. No observations have yet been made on mice lacking both netrins-1 and -3. Such mice would be expected to be deficient in the numbers of crest-derived cells that survive in the bowel. It is not clear how DCC-expressing cells in the outer gut mesenchyme escape the presumptive attraction of the source of netrin in the presumptive circular muscle layer to migrate toward the sources of netrin in the mucosa and pancreas. It is conceivable that the concentrations of netrins and the resulting gradients are patterned so as to favor migration toward the mucosa and pancreas. In addition, it is possible that netrins are presented to crest-derived cells more effectively in or near the mucosa than in the smooth muscle. The DCC expressed by the mucosa may facilitate its accumulation in this location.

In following a putative netrin gradient, the DCC-expressing subset of crest-derived cells must choose whether to migrate toward the mucosa or in the opposite direction toward the pancreas. It seems likely that only those crest-derived cells that express DCC as they migrate past the pancreatic buds are in a position to respond to the pancreatic netrin gradient. The remainder of the population would be expected to respond only to the mucosal source of netrin. The developmental regulation of DCC expression in the gut would also imply that the time during which crest-derived cells could be attracted to the pancreas is brief. The restrictions in time and space of the potential for attracting enteric crest-derived cells to migrate into the pancreas are likely to limit the loss of such cells from the gut and the number of neurons that ultimately arise in pancreatic ganglia.

Our observations strongly support the idea that netrins and their receptor, DCC, play critical roles in the development of the ENS and the innervation of the pancreas. Netrin/DCC signaling appears to be most crucial in the formation of the submucosal and pancreatic plexuses; however, netrin/DCC signaling may also play roles in the establishment of the extrinsic vagal innervation of the intestinal ganglia, the development of intraenteric connections, and the regulation of apoptosis.

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